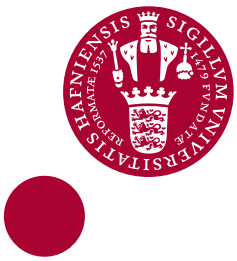

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Assessment of biochar and zero-valent iron for *in-situ* remediation of chromated copper arsenate contaminated soil

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Highlights

- Chromate copper arsenate (CCA) contaminated soils pose risks to the environment
- *In-situ* stabilization of CCA contaminated soil was tested with biochar and ZVI
- Soil remediation was evaluated based on chemical and microbiological techniques
- Biochar reduced bioavailable (Cu_{bio}), but not water-extractable Cu (Cu_{water})
- Combination of biochar and ZVI most effectively reduced toxic effects of CCA

Abstract

Chromated copper arsenates (CCA) have been extensively used as wood impregnation agents in Europe and North America. Today, CCA contaminated sites remain abundant and pose environmental risks that need to be properly managed. Using a TRIAD approach that combined chemical, ecotoxicological and ecological assessment of soil quality, we investigated the abilities of biochar and zero-valent iron (ZVI) to remediate CCA contaminated soil in a microcosm experiment. Soil samples from a highly contaminated CCA site (1364, 1662 and 540 µg g^{-1} of As, Cu and Cr, respectively) were treated with two different biochars (fine and coarse particle size; 1 % w w^{-1}) and ZVI (5 % w w^{-1}), both as sole and as combined treatments, and incubated for 56 days at 15 °C. In general, bioavailable As (As_{bio}) and Cu (Cu_{bio}) determined by whole-cell bacterial bioreporters corresponded well to water-extractable As and Cu (As_{water} and Cu_{water}). However, in biochar treatments, only Cu_{bio} and not Cu_{water} was significantly reduced. In contrast, under ZVI treatments only Cu_{water} and not Cu_{bio} was reduced, demonstrating the value of complementing analytical with bacterial bioreporter measurements to infer bioavailability of elements to soil microorganisms. The combined fine particle size biochar and ZVI treatment effectively reduced water extractable concentrations of Cr, Cu, and As on site by 45%, 45% and 43 % respectively, and led to the highest ecological recovery of the soil bacterial community, as measured using the [^{3}H]leucine incorporation technique. We conclude that the combined application of biochar and ZVI as soil amendments holds promise for *in-situ* stabilization of CCA contaminated sites.

Keywords

metal-contaminated soils, soil remediation, biochar, zero-valent iron, bioavailability, whole-cell biosensors

1. Introduction

Chromated copper arsenate (CCA) constitutes a mixture of Cr and Cu salts (CrO_3 and CuO) and arsenic acid (H_3As0_4) and has been extensively used as a wood preservative since the 1940s. In 2003, legislation in Europe and North America was passed which severely limited CCA application (European Commission, 2003; Humphrey, 2002; U.S. EPA, 2002), but a large number of CCA legacy contaminated sites remain (Bhattacharya et al., 2002; Hopp et al., 2006), with more than 100 sites in Denmark alone (Amternes Videncenter for Jordforurening, 1997; Nielsen et al., 2010).

CCA contaminated sites are of environmental concern as Cr, Cu and As pose severe risks to both human and environmental health. Arsenic is of particular concern due to its toxicity and mobility, by which it may also threaten surface and groundwater drinking water resources (Nielsen, 2013). In soils, As occurs most commonly as the inorganic oxyanions arsenite (As(III)) and arsenate As(V)) (Masscheleyn et al., 1991), with As(III) generally the most toxic and mobile form (Peters et al., 1996). However, Cu is less mobile, but may adversely affect ecosystem services provided by soil biota (Arthur et al., 2012; Nunes et al., 2016) and co-select for antibiotic resistance (Ashbolt et al., 2013; Song et al., 2017). Cr occurs in soil mostly as Cr(0), Cr(II), Cr(III) and Cr(VI), with Cr(III) the most stable and prevalent Cr redox species in most soils (Namiesnik and Rabajczyk, 2012), but the Cr(VI) species used in CCA formulations is considered substantially more toxic than reduced forms of Cr (Nielsen et al., 2010).

CCA and other multi-element contaminated soils can be remediated in various ways. Full remediation is often only possible by excavation, but due to the sheer number and size of contaminated sites worldwide, this approach is seldom feasible (Nielsen et al., 2016). Hence, *in-situ* stabilization by application of different soil amendments has been proposed as a more economical approach for reducing the mobility and toxicity of trace element contaminants (Bolan et al., 2014; Maurice et al., 2007), but the challenge remains to identify suitable soil amendments for

multi-element contaminated sites, as the individual elements may respond differently to soil treatments (Kumpiene et al., 2008; Qiao et al., 2018; Silvetti et al., 2014; Zhou and Haynes, 2010).

Biochar, a solid produced by pyrolysis of organic material, has gained particular interest as a cheap and effective amendment for remediation of cationic pollutants such as Cu, mainly by increasing pH and providing additional sorption sites (Beesley et al., 2011; Buss et al., 2012; Ippolito et al., 2012; Lehmann and Joseph, 2015). However, biochar may not work equally well for anionic trace element contaminants, as previous studies have observed an increase in bioavailability and mobility of As (Beesley et al., 2010; Hartley et al., 2009; Kim et al., 2018; Wang et al., 2017). Rather, iron-bearing compounds such as zero-valent iron (ZVI) have proven more useful for *in-situ* stabilization of As (Kumpiene et al., 2006; Miretzky and Cirelli, 2010; Nielsen et al., 2011). Sneath et al. (2013) proposed a combination of biochar and ZVI as a promising amendment for *in situ* stabilization of soil contaminated with complex mixtures of metals, arsenic and organics. Several recent studies investigated the synthesis and characterization of iron-coated biochar for metal(loid) removal from aqueous media and found enhanced sorption of Cr(VI) (e.g. Zhu et al., 2018; B. Wu et al., 2018; Diao et al., 2018), Cu (e.g. Kolodynska and Bak, 2018; Yang et al., 2018b) as well as As (e.g. Bakshi et al., 2018; He et al., 2018; Zhou et al., 2014). However, the combination of biochar and iron as a treatment for remediation of multi-element contamination of soils and other media is less well studied (Lu et al., 2018).

Consequently, the aim of the present study was to perform a soil microcosm study to understand both the independent and combined efficiency of biochar and ZVI for stabilizing CCA contaminated soil. Remediation treatment effects were assessed after 1, 7, 28 and 56 days using a soil quality TRIAD approach. The TRIAD approach combines investigations of soil chemistry (exposure and bioavailability), (eco)toxicology, and ecology as recently recommended for site-specific risk assessment of contaminated soils (ISO 19204, 2017). Exposure and bioavailability were assessed by a combination of chemical analyses (ICP-OES, trace element speciation analysis etc.) and of bioluminescent whole-

cell bacterial bioreporters responding specifically to bioavailable Cu and As, respectively. Soil toxicity was assessed in a laboratory bioluminescence inhibition assay with a whole-cell bacterial bioreporter. Soil ecology was studied by following the recovery of growth in indigenous soil bacteria as measured by the [^{3}H]leucine incorporation technique, providing a proxy for secondary bacterial productivity (Brandt et al., 2015). We hypothesized that a combination of ZVI and biochar would be more efficient in reducing environmental risks posed by Cr, Cu and As in CCA contaminated soil than independent applications of ZVI and biochar. In addition, we hypothesized that the high surface area of fine biochar would stabilize multiple elements more efficiently than coarse biochar.

2. Materials and Methods

2.1. CCA contaminated soil and experimental soil amendments

Contaminated soil was collected from the upper 20 cm of a former wood impregnation site north of Copenhagen, Denmark (sampling point: 55°57'19.7"N 12°21'18.1"E, station L4), highly contaminated with Cr, Cu, and As (see Nielsen et al., 2011, for detailed site description and Tardif et al., 2019, for detailed physico-chemical characterization). The texture was classified as loamy sand with 39.9 % coarse sand (200-2000 µm), 44.6 % fine sand (20-200 µm), 11.0 % silt (2-20 µm), and 4.5 % clay (< 2 µm) and had an initial gravimetric moisture content of 17.0 %. After sampling, soil was placed under a fume hood for air-drying, sieved (2 mm mesh) and stored in plastic buckets until setup of soil microcosm experiments. General soil characteristics are summarized in Table 1.

Biochar in two different particle sizes was produced from *Miscanthus x giganteus* (charring at 850°C for 30 min; PYREG, Dörth, Germany): coarse biochar, pieces of max. 0.3 mm diameter and 20 mm length, and fine biochar, milled to a particle size of < 2 mm. Biochar characteristics can be found in Table 1; see Bamminger et al. (2016) for full details. Microscale zero-valent iron (ZVI) (Ferox-Flow, Hepure, New Jersey, USA) had a particle size ranging from 45 – 150 µm and contained up to 2.5 %

carbon and 2 % silicon. The ZVI powder had been stored for about 1.5 years previous to use and therefore its reactivity may have been somewhat reduced compared to fresh ZVI.

2.2. Chemical characterization of soil and biochars

The elemental composition of both soil and biochars were analyzed by inductively coupled plasma optical emission spectroscopy (Agilent Technologies 5100 ICP-OES 16/6-16, Agilent Technologies Inc., Santa Clara, USA) following microwave digestion of 0.2 g pulverized dried material with 5 ml conc. HNO_3 and 1 ml of 15 % H_2O_2 in a Milestone Ultrawave microwave (Single Reaction Chamber Microwave Digestion System). pH of soil and biochar was measured in deionized water suspension, the former in a soil:water ratio of 1:2.5 (w v^{-1}) and the latter in a biochar:water ratio of 1:12.5 (w v^{-1})) due to its low material density. Measurement was performed with a 913 pH meter (Metrohm, Herisau, Switzerland) after manual shaking and sedimentation for at least 30 min. Soil texture was analyzed by a combined sedimentation and sieving method as described by Borggaard et al. (2011).

2.3. Experimental design

Replicate soil microcosms (n = 4, except n = 6 for control treatment) were each prepared with 200 g air dried soil in polypropylene centrifuge bottles (250 mL). The following experimental treatments were established by mixing the soil homogeneously with biochar and ZVI, in the following combinations: control (CCA contaminated soil only), BC_{coarse} (CCA contaminated soil + 1 % w w^{-1} coarse biochar), BC_{fine} (CCA contaminated soil + 1 % w w^{-1} fine biochar), ZVI (CCA contaminated soil + 5 % w w^{-1} ZVI), BC_{coarse} + ZVI (CCA contaminated soil + 1 % w w^{-1} coarse biochar + 5 % w w^{-1} ZVI), and BC_{fine} + ZVI (CCA contaminated soil + 1 % w w^{-1} fine biochar + 5 % w w^{-1} ZVI). Subsequently, the soils were rewetted with 20 mL Milli-Q water (Time 0), resulting in gravimetric water content of 10.0, 9.9, 9.5, and 9.4 % for control, biochar only, ZVI only, and the combined treatments respectively, based on dry weight of soil plus amendments. Microcosms were sealed with perforated parafilm and incubated at 15 °C in the dark for up to 56 days. Soil moisture was adjusted every two to four days to ensure a

maximum deviation from initial moisture content of ± 0.9 % (absolute). Soil subsamples (7 g) were collected from each microcosm after 1, 7, 28, and 56 days and immediately extracted and analyzed for chemical and microbiological properties (see subsequent sections below).

2.4. Soil extraction for chemical and whole-cell bioreporter analyses

Each microcosm soil sample was extracted with 35 mL Milli-Q water (i.e. soil:water-ratio 1:5) in 50 mL Falcon tubes by shaking for 2 hours at 200 rpm in a horizontal position followed by centrifugation at 10000 g for 20 minutes. The resulting supernatant was analyzed for total water-extractable As (As_{water}), bioavailable Cu (Cu_{bio}), bioavailable As (As_{bio}), As redox speciation (As(V) and As(III)), pH, and dissolved organic carbon (DOC). Samples from the last sampling day were additionally analyzed for water-extractable total Al, As, Ca, Cr, Cu, Fe, K, Mg, P, and Zn as described below.

2.5. Chemical characterization of the soil-water extracts

As_{water} was determined by GF-AAS (PinAAcle 900Z Atomic Absorption Spectrometer, PerkinElmer, Waltham, Massachusetts, USA) on acidified subsamples of the extract (0.2 % HNO_3). DOC concentrations were measured with a Shimazdu TOC-VPN-analyser (Shimadzu Corp., Kyoto, Japan) on a subsample of the supernatant that had been passed through a 0.45 µm cellulose acetate filter (Q-Max RR syringe filters, Frisenette, Denmark) that had been stored at 4 °C for a maximum of 4 weeks. pH was analyzed on all extracts as described for the general soil characterization (2.2).

For samples collected at the end of the experiment (Day 56), water-extractable total Al, As, Ca, Cr, Cu, Fe, K, Mg, P and Zn were analyzed in the water-extract (3.5 % HNO_3 acid concentration, stored at room temperature) by ICP-OES.

2.6. Whole-cell bacterial bioreporter assays for bioavailable Cu, As and soil toxicity

Cu_{bio} was measured with *Pseudomonas fluorescens* DF57-Cu15 while *P. fluorescens* DF57-Cu40E7 was used as a constitutive control strain for taking potential sample matrix effects (e.g. masking of emitted light) into account (Brandt et al., 2008; Tom-Petersen et al., 2001). Bioavailable Cu was operationally defined as Cu species that were able to induce expression of Cu-regulated *luxAB* genes in the employed *P. fluorescens* DF57-Cu15 bioreporter within a 1.5 h incubation period. Both analysis and subsequent calculations were performed according to Brandt et al. (2008), except for final re-suspension of the biosensor cells, which was performed in a MOPS-buffered minimal medium with a low capacity for Cu-complexation (see Supporting Information **Error! Reference source not found.**).

Analogously, As_{bio} was determined with *Escherichia coli* pJAMA arsR (Stocker et al., 2003), and *E. coli* pUCD 607 HB101 (Rattray et al., 1990) as a constitutive control strain. Thereby, As bioavailability was operationally defined as As species that were able to induce expression of arsenite-regulated *luxAB* genes in the utilized *E. coli* pJAMA arsR bioreporter within a 2 h incubation period. The bioreporter strain possesses an arsenate reductase and responds to both inorganic As(V) and As(III). Therefore, the bioreporter analysis was carried out immediately after the extraction in order to minimize possible changes in arsenic speciation. Details on the bioassay as well as calibration by As-speciation data can be found in Supporting Information B, C and Figure S1.

Although As_{bio} and As_{water} gave, on average, similar results, in this paper we focus our data presentation and analysis on the As_{water} data due to the comparably higher precision of this method (Supporting Information, Figure S2). Likewise, Cu_{bio} and Cu_{water} yielded results in the same range, with about 92 % of Cu_{water} actually being bioavailable (Figure 2B), but in this case the bioreporter data was of sufficient quality; we thus used both Cu_{bio} and Cu_{water} for data presentation and analysis.

E. coli pUCD 607 HB101 was also used as a stand-alone toxicity assay in the soil-water extracts in order to assess the ecotoxicological effects on an introduced test organism. For all bio-assays,

bioluminescence was recorded on a plate reader (FLUOStarOptima, BMG Labtech, Ortenberg, Germany) after addition of decanal as described previously (Nybroe et al., 2008).

2.7. Bacterial growth

Bacterial growth (i.e. heterotrophic productivity of indigenous soils bacteria) was measured using the [^{3}H]leucine incorporation microcentrifugation technique (Bååth et al., 2001). Briefly, bacteria were extracted from soil (1 g fresh wt) with 10 mL Milli-Q water on a multi-shaker at highest speed for three minutes. Following centrifugation (1000 × g, 10 min), 1.5 mL aliquots of the resulting soil bacterial suspensions (supernatants) were amended with 50 µl of [^{3}H]-labelled leucine (6.4 kBq per 50 µL) to yield a final leucine concentration of 200 nM. Incubations were terminated after either 1 or 2 hours by adding 50 % trichloroacetic acid (TCA). Bacteria in dead controls were killed with 50 % TCA prior to addition of [^{3}H]leucine. Finally, the incorporated [^{3}H]leucine was physically separated from non-incorporated [^{3}H]leucine via a series of centrifugation and washing steps (Bååth et al., 2001) and radioactivity was measured by scintillation counting (Tri-Carb 2910 TR, Perkin-Elmer, USA). Results were normalized using the mean growth rate of the control at Day 1.

2.8. Statistical analysis

Statistical analyses were performed using R (R version 3.3.1, The R Foundation for statistical computing, 2016) and Microsoft Office Excel (Version 14.0.7166.5000, Microsoft Office Home and Student, 2010). The *lmer*-function was used to perform regression analysis on As_{water}, As_{bio}, Cu_{bio}, toxicity, and [^{3}H]leucine incorporation data. The general model included the interaction between the two treatments (biochar and ZVI), considering time as fixed effect and the microcosms as random effects. Model validation was performed by qq-plotting and Shapiro-Wilk-Normality test. Except for As_{water}, all statistical analyses were performed on log-transformed data. Non-significant factors and interactions were excluded from the model by *step*-function. *Contrast*-function within the *lsmeans*-

185 package was used for deriving p-values for pairwise comparisons. P-value adjustment for multiple
186 comparisons was performed according to the Holm-Bonferroni-method, which controls family-wise
187 error rate (Holm, 1979). For the data from the ICP-OES analysis (water-extractable total metals at the
188 last sampling time), two-way ANOVA (*aov*-function) was performed, since no time effect had to be
189 considered. As for the other analyses, the Shapiro-Wilk Test was performed to check for normality.
190 Only analysis of Cr-data was performed on log-transformed data, while raw data was used for all other
191 elements. P-values for statistically significant differences between the treatments were derived using
192 TukeyHSD test. Throughout, a significance level of $p < 0.05$ was applied.

193 # 3. Results

194 *3.1 Treatment effects on soil chemical properties and bioavailability of As and Cu*

195 In general, the combined treatment of ZVI with BC_{fine} was most effective in reducing As_{water}, Cu_{water}, and
196 Cr_{water} by 43 %, 45 %, and 45 %, respectively, compared to the control and measured after 56 days
197 (Figure 1). Looking into the effects of the separate treatments, ZVI alone also reduced the three
198 elements, but to a lesser extent: As_{water} was reduced by 27 % while Cu_{water} was reduced by 29 %
199 compared to the control ($p < 0.001$). In addition, Cr was significantly reduced by ZVI alone by 39 %,
200 resulting in a concentration of 0.40 µg g^{-1} dry soil ($p < 0.05$). Biochar alone did not have a significant
201 effect on the extractability of any of the elements. Nevertheless, biochar affected the bioavailability of
202 Cu (Figure 2). At Day 1 and Day 7, Cu_{bio} was significantly reduced by BC_{fine}, ($p = 0.014$ and $p = 0.004$,
203 respectively) (Figure 2A). This was also observed at the end of the incubation period, but the effect
204 was not statistically significant. In contrast to Cu_{water}, which was reduced by ZVI as mentioned above,
205 Cu_{bio} was not affected by ZVI alone (Figure 2B).
206 Soil amendments also affected other chemical soil properties. On average, treatment with biochar
207 increased pH from 6.4 ± 0.04 to about 6.7 ± 0.07 both with and without ZVI (Day 1). DOC decreased
208 after the first sampling and generally was very low (< 8 mg L^{-1}) (data shown in supporting information,

Figure S3). As there was an analytical problem with some of the blanks, interpretation of the data must be treated cautiously; however, it seems that both biochar and ZVI slightly reduced DOC (except for sampling at Day 56).

Treatments with biochar and/or ZVI also affected other elements, as summarized in Table 2. Biochar significantly increased water extractable fractions of P and K ($p < 0.01$ and $p < 0.001$), and significantly reduced possible toxic elements such as Al and Zn ($p < 0.001$). ZVI enhanced the reductions in Al and Zn content in the extract; however, it also reduced P. In addition, ZVI strongly reduced extractable Fe and Mg.

In general, soil amendment effects on soil chemistry were remarkably stable over time. Hence, As_{water} (Supporting Information, Figure S2), Cu_{bio} (Figure 2A), pH (data not shown) and As speciation (Supporting Information, Figure S1) did not exhibit any statistically significant changes during the experimental period of 56 days.

3.2. Effects of soil amendments on soil toxicity

Treatments did not have any consistent effect on toxicity of the soil-water extracts as assessed by the bioreporter assay with *E. coli* pUCD 607 HB101 (Supporting Information, Figure S4). ZVI increased the relative response of the bioreporter; however, the effect was only statistically significant at Day 7. No general trends over time could be observed.

3.3. Treatment effects on bacterial growth rate

[^{3}H]leucine incorporation increased markedly from Day 1 to the subsequent sampling days (Figure 3). At the last two sampling times (Day 28 and Day 56), biochar significantly increased bacterial growth rate irrespective of whether or not ZVI was present ($p < 0.001$ at Day 28, $p < 0.005$ at Day 56). ZVI slightly increased [^{3}H]leucine incorporation as well, but the effect was not statistically significant. These effects could not be seen at the earlier samplings; instead, the opposite was observed: At Day

1, biochar significantly reduced bacterial growth rate compared to the treatments without biochar ($p < 0.001$).

4. Discussion

4.1. Effects of soil amendments on soil quality

To the best of our knowledge, our study is the first to investigate *in-situ* stabilization with both biochar and ZVI for remediation of a CCA-contaminated soil and to assess soil quality recovery using a TRIAD approach with three lines of evidence: chemistry, (eco)toxicology, and ecology. In our study, the (eco)toxicology line of evidence (bioluminescence inhibition assay) was insensitive to changes imposed by the experimental treatments and soil quality was best assessed by the other two lines of evidence. Our study thus demonstrates the value of using the [^{3}H]leucine incorporation assay as a sensitive ecological indicator of soil quality recovery and argues against sole reliance on classical ecotoxicological short-term assays with introduced test organisms. A pronounced increase in bacterial growth during the first weeks of the study was observed for all treatments, including the control, and was certainly a rewetting effect (Meisner et al., 2015), as the soil had been air-dried prior to setting up the microcosm experiment.

As hypothesized, we found that fine biochar in combination with ZVI was the most effective treatment to significantly decrease the risks posed by As, Cu and Cr. Hence, chemical and ecological lines of evidence consistently indicated that the combination of BC_{fine} and ZVI was the most effective treatment for reducing exposure and bioavailability of metals (Figures 1 and 2) and restoring the ecological functionality of the soil (Figure 3). The latter claim is based on the assumption that an increase in secondary bacterial productivity as measured by the [^{3}H]leucine incorporation technique is indicative of ecological recovery. Generally, treatments with milled biochar (BC_{fine}) have invoked a greater effect as compared with coarse biochar, likely due to its provision of additional sorption sites due to smaller

255 particle size and increased surface area. Nevertheless, the observed treatment effects were moderate
256 and As and Cu concentrations in the water extract (Figure 1) still exceeded Danish groundwater quality
257 criteria, by 185-fold and approximately 4-fold, respectively (Danish EPA, 2002). These were chosen as
258 reference criteria because no general guidelines for water-extractable concentrations exist. Other
259 studies that have explored the use of novel iron-modified biochars have found them to be comparable,
260 or even more effective at reducing extractable fractions of As (Qiao et al., 2018; C. Wu et al., 2018), Cu
261 (Yang et al., 2018a), or Cr (Lyu et al., 2018; Mandal et al., 2017; Zhang et al., 2017); however, none of
262 them looked into the combined effect on CCA-contaminated soil.

263 Regarding the isolated effect of ZVI, our study is consistent with previous soil studies demonstrating
264 the ability of ZVI to stabilize Cr, Cu and As (Kumpiene et al., 2006; Nielsen et al., 2016, 2011; Sneath et
265 al., 2013). With respect to As specifically, our reported two-fold decrease in water-extractable As is
266 consistent with Sneath et al. (2013), but much lower than the 93 and 98 % reductions in leachable As
267 reported by other aforementioned studies. These findings suggest that the efficacy of the remediation
268 treatment is highly dependent on the quality and specific properties of the amendment. We also found
269 that while water-extractable Cu decreased after treatment with ZVI, albeit less strongly than seen in
270 the above studies, bioavailable Cu did not. Similarly, Kumpiene et al. (2006) reported a decrease in
271 pore water concentrations of Cu after treatment with ZVI, but pointed out that ZVI did not reduce
272 bioaccessible Cu (determined by sequential extraction) and in fact, even increased plant uptake.
273 Generally, we observed a low water-extractability of Cr, likely because Cr at this site was reported to
274 be present almost exclusively in the form of Cr(III) (Nielsen et al., 2016) and primarily associated with
275 hard-to-extract Fe oxides in the oxic top soil (Tardif et al., 2019). In accordance with others, we also
276 observed a significant reduction in water-extractable Cr upon addition of ZVI (Nielsen et al., 2011;
277 Sneath et al., 2013; Zhang et al., 2018), probably due to provision of additional sorption sites on Fe-
278 (hydr)oxide surfaces for Cr(III) (Fendorf, 1995; Kumpiene et al., 2006). Concomitant with the effects of

279 ZVI on soil chemistry, ZVI amendment reduced toxicity only slightly as indicated by the *E. coli* pUCD
280 607 HB101 biosensor assay and did not significantly increase [^{3}H]leucine incorporation rates.

281 Biochar amendments alone did not substantially reduce concentrations of water-extractable elements,
282 in contrast to earlier findings (Beesley et al., 2014; Mitchell et al., 2018; Sneath et al., 2013; Uchimiya
283 et al., 2011a), but as expected, biochar did significantly reduce Cu bioavailability. Likewise, a previous
284 study found that biochar addition to a sandy soil did not alter water-extractable Cu although it did
285 reduce Cu phytoavailability (Namgay et al., 2010). Our study thus demonstrated that although biochar
286 was generally less effective than ZVI in reducing water-extractable concentrations of the contaminants,
287 it was more effective in reducing bioavailable Cu. This may possibly explain why bacterial growth was
288 more effectively restored with biochar than under ZVI treatment (Figure 3). Hence, Cu most likely
289 represented the most toxic element in the studied CCA contaminated soil (Tardif et al., unpublished
290 results) and bioavailable Cu determined with the *P. fluorescens* bioreporter used here has previously
291 been shown to constitute an excellent predictor of Cu toxicity effects in soil bacterial communities
292 (Nunes et al., 2016; Song et al., 2017). Our findings are also in accordance with a recent study showing
293 that biochar amendment restored microbial activity in Cu contaminated soil (Moore et al., 2018). A
294 number of factors, such as soil properties (e.g. pH, texture, or CEC (Uchimiya et al., 2011a)), biochar
295 properties (e.g. pyrolysis temperature (Uchimiya et al., 2011b)), redox conditions (El-Naggar et al.,
296 2018), and/or Cu-concentrations in soil (Ippolito et al., 2012; Lu et al., 2017; Mackie et al., 2015)
297 influence Cu water extractability and bioavailability in soil after biochar addition. As soil DOC was very
298 low and soil pH was near neutral in our study, it is plausible that biochar influences were relatively
299 small and not as pronounced as seen in studies with more acidic soils (Beesley et al., 2014; Uchimiya
300 et al., 2011a). In contrast to previous literature (Choppala et al., 2015), we did not see any effect of
301 biochar on Cr_{water}. This is likely to have been due to a high fraction of the total Cr having already been
302 reduced and stabilized in soil oxide minerals (Nielsen et al., 2016, 2011; Tardif et al., 2019) (see above
303 discussion on effects of ZVI) and therefore, no additional effects of the treatment could be observed.

Although our study cannot provide clear predictions of long-term field applicability of ZVI and biochar amendments for remediation of CCA contaminated soils, it demonstrated that treatment effects on soil chemical parameters remained quite stable over time. The effects of remediation treatments on both Cu and As bioavailability were detectable after only one day, indicating that oxidation and sorption reactions must have occurred during the first 24 hours, as previously shown (Jain et al., 1999; Nielsen et al., 2011). Long-term efficacy of ZVI has been previously suggested by Tiberg et al. (2016) who argued that the effect of ZVI on As leaching and mobility should remain stable over at least up to 15 years. In contrast, the efficacy of biochar may be more short-lived, as shown in our study by the fact that significant reduction in Cu bioavailability (Cu_{bio}) was only observed during the first week. This raises the question of whether or not the observed biochar effect would diminish over time. Also, organic acids released from plants grown at a site could potentially lead to a release of Cu from biochar (Oustriere et al., 2017), which would be problematic, since long-term stability of the amendments is crucial for remediation. Furthermore, flooding, which was frequently observed at this study site, could be problematic; under anaerobic conditions biochar has been shown to enhance As mobilization, possibly by acting as an electron shuttle (Kappler et al., 2014; Wang et al., 2017). This was shown to be of particular concern for biochar produced at high pyrolysis temperatures (Beiyuan et al., 2017).

4.2. Comparison of chemical and microbiological measurements of bioavailability of Cu and As

Assessment of bioavailable element fractions depends on the underlying definition of "bioavailability" (Semple et al., 2004) and should not be assessed solely by chemical analysis (Kumpiene et al., 2017; Touceda-Gonzalez et al., 2017). Since *in-situ* stabilization of contaminated sites relies on reducing the bioavailability of contaminants, we complemented soil chemical analysis with whole-cell biosensors specific for both As and Cu, but not for Cr, as no biosensor was available. In our study, biosensor analysis generally yielded concentrations in the same range as chemical analysis, implying that all dissolved Cu and As species remained bioavailable (Supporting Information, Figure S2 and Figure 2). This suggests that under the given conditions, water-extractable fractions of both, As and Cu roughly

329 reflected the bioavailable, and thus toxicologically most relevant fractions. Nevertheless, the deviation
330 between As_{water} and As_{bio} appeared rather random and could not be explained by any systematic effect,
331 but instead likely revealed the sensitivity of the bioassay to other soil properties. Specific interferences
332 with the As biosensor could be changes in As redox speciation, presence of other elements such as Fe
333 or P (e.g. Kuppardt, 2010; Trang et al., 2005), influence of co-contaminants, and other general matrix
334 effects such as changes in pH. Hence, we suggest that further optimization of the As biosensor assay
335 for contaminated soils is needed. On the other hand, Cu_{bio} measured with the Cu biosensor did not
336 significantly exceed Cu_{water}, but instead showed a consistent trend in the effects of the treatments on
337 Cu_{bio}. These findings suggest that the Cu biosensor reliably assessed Cu bioavailability even in the
338 studied multi-element contaminated soil (Figure 2). Consistent with previous studies, which showed
339 even larger deviations between Cu_{bio} and Cu_{water} especially for samples with higher DOC content
340 (Brandt et al., 2008; Maderova et al., 2011; Nybroe et al., 2008), findings from our study further
341 confirm the usefulness of complementing chemical measurements with bacterial biosensor data to
342 infer Cu bioavailability in contaminated soils.

343 *4.3. Concluding remarks*

344 We conclude that combined application of biochar and ZVI as soil amendments holds promise for *in-*
345 *situ* stabilization of CCA contaminated sites and for the ecological recovery of soil microbiota at these
346 sites. As treatment effects were less pronounced than in other studies, we suggest that future attempts
347 to use these types of amendments for *in-situ* stabilization of multi-element contaminated sites should
348 focus on the quality and properties of biochar, ZVI, and novel iron-biochar composites to ensure
349 optimization of remediation. Further research should focus on the long-term stability of these
350 amendments, their field applicability, and on replicability of the results with other types of biochar and
351 different soils. More studies are needed to evaluate whether the achieved improvement in soil quality
352 is sufficient to enable plant growth, and thereby to serve, as suggested previously (e.g. de Oliveira et
353 al., 2017; Sneath et al., 2013), as a first step towards further remediation.

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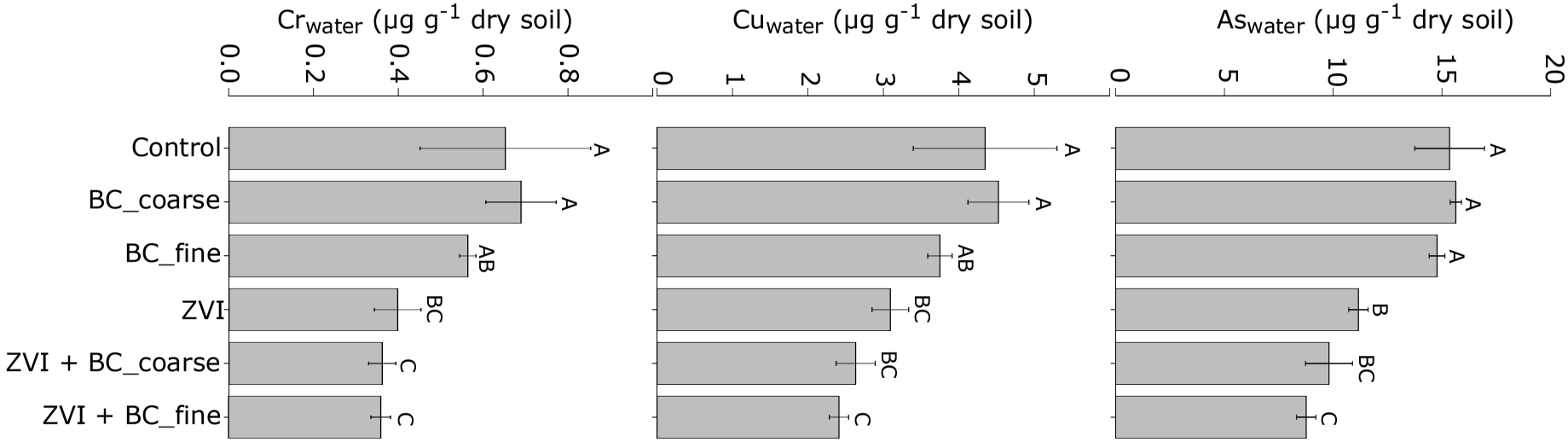
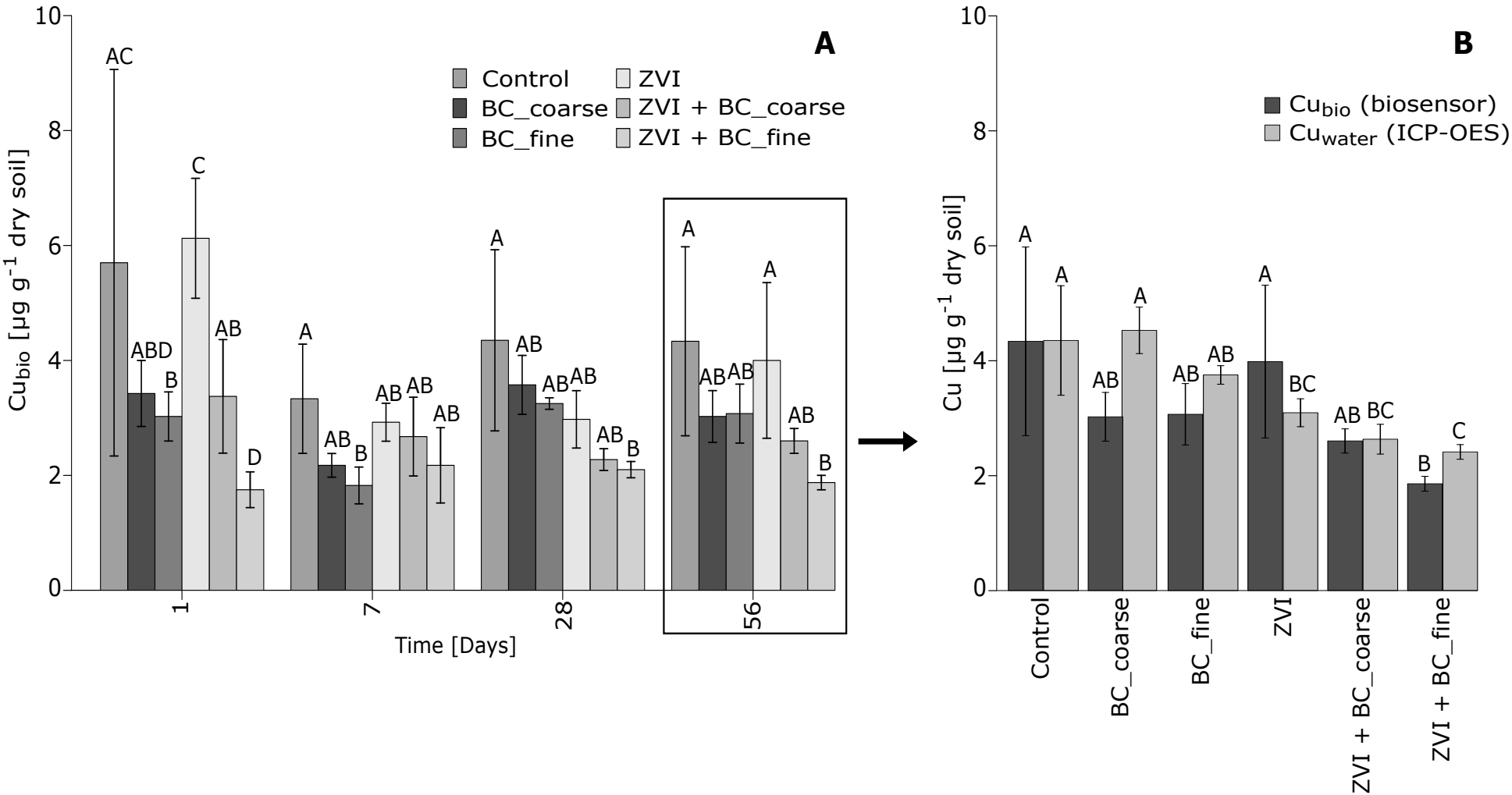
Figure legends.

Figure 1. Total water-extractable arsenic (As_{water}), copper (Cu_{water}) and chromium (Cr_{water}) after 56 days of incubation, measured with ICP-OES (n = 4 except for control, n = 6). Error bars represent standard deviations; different letters indicate statistically significant differences between treatments at $p < 0.05$. BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron.

Figure 2. (A) Bioavailable copper (Cu_{bio}) measured by Cu biosensor analysis with *Pseudomonas fluorescens* DF57 Cu15 (n=4 except for control, n=6). Error bars represent standard deviations; different letters indicate statistically significant differences between the treatments ($p < 0.05$). BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron. (B) Comparison of bioavailable copper (Cu_{bio}) and total water-extractable copper (Cu_{water}) after 56 days of incubation. (n=4 except for control, n=6). Error bars represent standard deviation; different letters indicate statistically significant differences between the treatments ($p < 0.05$); statistical analysis was performed separately for Cu_{water} and Cu_{bio}, meaning that different letters cannot be used for comparison between Cu_{water} and Cu_{bio}). BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron.

Figure 3. [^{3}H]leucine incorporation, normalized by the mean of the control at Day 1 (n=4 for non-control treatments; n=6 for control treatment except for control at Day 7, n = 5). Error bars represent standard deviations; different letters indicate statistically significant differences between treatments within one sampling time at $p < 0.05$. BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron.


Cr$_{water}$ (µg g^{-1} dry soil)
0.0
0.2
0.4
0.6
0.8
Cu$_{water}$ (µg g^{-1} dry soil)
0
1
2
3
4
5
As$_{water}$ (µg g^{-1} dry soil)
0
5
10
15
20
Control
BC_coarse
BC_fine
ZVI
ZVI + BC_coarse
ZVI + BC_fine
A
A
AB
BC
C
C
A
A
AB
BC
BC
C
A
A
A
B
BC
C


A
Control
BC_coarse
BC_fine
ZVI
ZVI + BC_coarse
ZVI + BC_fine
Cubio [µg g-1 dry soil]
Time [Days]
1
7
28
56
AC
ABD
B
C
AB
D
A
AB
B
AB
AB
AB
A
AB
AB
AB
AB
B
A
AB
AB
A
AB
B
B
A
A
A
AB
AB
A
BC
AB
BC
B
C
Cu [µg g-1 dry soil]
Cubio (biosensor)
Cuwater (ICP-OES)
Control
BC_coarse
BC_fine
ZVI
ZVI + BC_coarse
ZVI + BC_fine

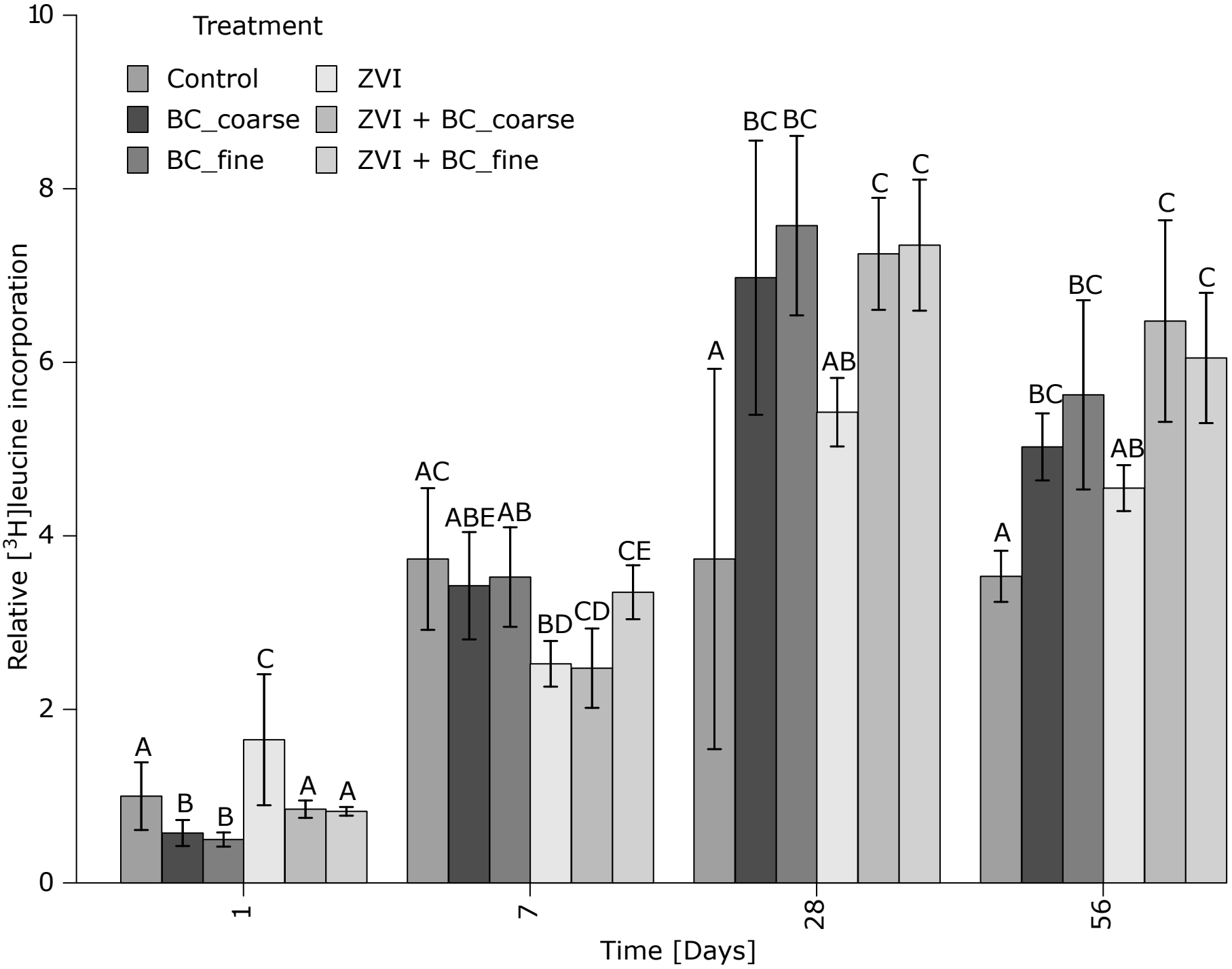

Treatment
Control
BC_coarse
BC_fine
ZVI
ZVI + BC_coarse
ZVI + BC_fine
Relative [3H]leucine incorporation
Time [Days]
0
2
4
6
8
10
1
7
28
56
A
B
B
C
A
A
AC
ABE
AB
BD
CD
CE
A
BC
BC
AB
C
C
A
BC
BC
AB
C
C

Table 1: Soil and biochar characteristics.

	C_{org}	N_t	pH [H_2O]	Al	As	Cr	Cu	Fe	Pb	Zn
	%	%		[µg g^{-1}]						
Soil	2.3	<LOD	6.4	7974	1364	540	1662	10580	29	120
Biochar	77.44	0.58	10.1	911	9	8	50	993	5	69

Table 2: Treatment effect on water-extractable elements after 56 days of incubation. (n = 4 except for control, n=6). Results are presented as means ± std. Different letters indicate statistically significant differences at $p < 0.05$). BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron.

[µg g^{-1}]	Control	BC_{coarse}	BC_{fine}	ZVI	ZVI + BC_{coarse}	ZVI + BC_{fine}
Al	88.27 ± 7.16 ^{A}	67.49 ± 3.86 ^{BC}	59.34 ± 2.69 ^{BD}	75.07 ± 2.16 ^{C}	51.45 ± 3.82 ^{DE}	46.94 ± 1.19 ^{E}
Ca	21.16 ± 0.91 ^{A}	18.83 ± 0.44 ^{B}	19.11 ± 0.15 ^{B}	20.58 ± 0.18 ^{A}	18.07 ± 0.36 ^{B}	18.21 ± 0.33 ^{B}
Cr	0.65 ± 0.20 ^{A}	0.69 ± 0.08 ^{A}	0.56 ± 0.02 ^{AB}	0.40 ± 0.05 ^{BC}	0.36 ± 0.03 ^{C}	0.36 ± 0.02 ^{C}
Fe	19.93 ± 6.16 ^{AB}	24.63 ± 2.38 ^{A}	21.38 ± 1.76 ^{A}	13.22 ± 0.98 ^{B}	13.90 ± 0.92 ^{B}	13.60 ± 0.85 ^{B}
K	51.10 ± 1.88 ^{A}	121.50 ± 2.26 ^{B}	123.88 ± 3.11 ^{B}	47.51 ± 0.86 ^{A}	108.79 ± 1.67 ^{C}	112.34 ± 3.12 ^{C}
Mg	4.98 ± 0.89 ^{A}	5.74 ± 0.39 ^{A}	5.41 ± 0.21 ^{A}	3.97 ± 0.16 ^{B}	4.09 ± 0.12 ^{B}	4.14 ± 0.09 ^{B}
P	0.90 ± 0.17 ^{A}	1.39 ± 0.21 ^{B}	1.43 ± 0.07 ^{B}	0.62 ± 0.07 ^{A}	0.86 ± 0.16 ^{A}	0.75 ± 0.14 ^{A}
Zn	0.49 ±0.05 ^{A}	0.37 ± 0.03 ^{BC}	0.31 ± 0.01 ^{B}	0.40 ± 0.01 ^{C}	0.25 ± 0.02 ^{D}	0.23 ± 0.01 ^{D}

Supporting information for

Assessment of biochar and zero-valent iron for *in-situ* remediation of chromated copper arsenate contaminated soil

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A. MOPS-buffered minimal medium for Cu-Biosensor analysis

After harvesting the overnight culture of the Cu-biosensors, the cells were re-suspended in autoclaved MOPS-buffered minimal medium. Resuspension only took place immediately before addition to the microtiter plate. The medium consists of the following:

- 100 mM KCL
- 20 mM MOPS (pH 7.2 buffer)
- 7.6 mM $(NH_4)_2SO_4$
- 4 mM glycerophosphate

➔ pH adjustment to 7.2 was performed with NaOH

➔ after autoclaving (at the day of use), glucose was added to a final concentration of 0.8 % (w v^{-1})

B. Bioassay

E. coli bioreporter cells from both pJAMA arsR as well as pUCD 607 HB101 were revived from cryo stocks on LB agar plates with 50 µg L^{-1} ampicillin incubated overnight at 37 °C. One single colony was transferred to 25 ml LB + 50 µg L^{-1} ampicillin and grown overnight at 37 °C at 200 rpm in a horizontally positioned in 50 mL Falcon tubes. Bioreporter cells in the overnight cultures were harvested at 5000 g for 10 minutes and re-suspended in LB to a final OD_{600} of 0.02. Bioreporter cell suspension (100 µL) was subsequently mixed with 100 µL of As standard solution or sample solution in white 96-well microtiter plates (NUNC™, ThermoFisher Scientific Inc., Massachusetts, USA) and incubated for 2 hours at 28 °C. As standards were prepared with both As(V) ($Na_2HAsO_4 \times 7\ H_2O$, 0 – 40 µM range) and As(III) ($NaAsO_2$, 0 – 4 µM range) on each microtiter plate. Finally, As(V) standard calibration data was used to calculate As_{bio}, justified by results of As redox speciation analysis revealing that on average less than 2.1 % of the total As concentration in the extracts occurred as As(III) (see supporting information SI C and Figure S 2 for both As speciation procedure and results).

C. Arsenic speciation analysis

Arsenic speciation was performed using disposable selective cartridges (MetalSoft Center, NY, USA) which retain arsenate (As-V) while arsenite (As-III) passes through. Water extracts were diluted 10 times in order to not exceed the capacity of the cartridges and passed through the cartridge with a syringe at a speed of 60 ± 30 mL per minute, whereby the first 5 mL were discarded (Krogsriis, 2006; Meng et al., 2001). The following 3 mL of filtrate were collected and acidified with nitric acid (0.2 % final acid concentration) and stored at 4 °C until subsequent analysis on graphite-furnace atomic absorption spectroscopy (GF-AAS, PinAAcle 900Z Atomic Absorption Spectrometer, PerkinElmer, Waltham, Massachusetts, USA). Separation of the species by the cartridge was performed as soon as possible after extraction, at least within 5 hours. Results were compared with results for As_{ext}, giving the total As concentration before the cartridge.

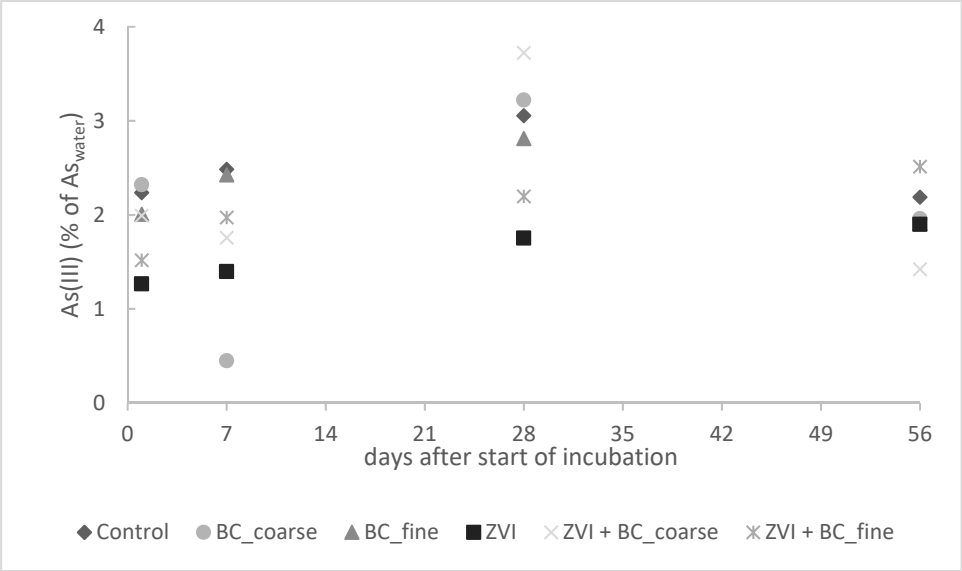

Figure S1: Arsenite (As(III)) content [%] relative to water-extractable arsenic (As_{ext}) (GF-AAS). Measurements were taken before and after passage through a selective speciation cartridge. (n = 4, for control n = 6). (BC = biochar; “coarse” and “fine” refer to different particle sizes of the biochar; ZVI = zero-valent iron)

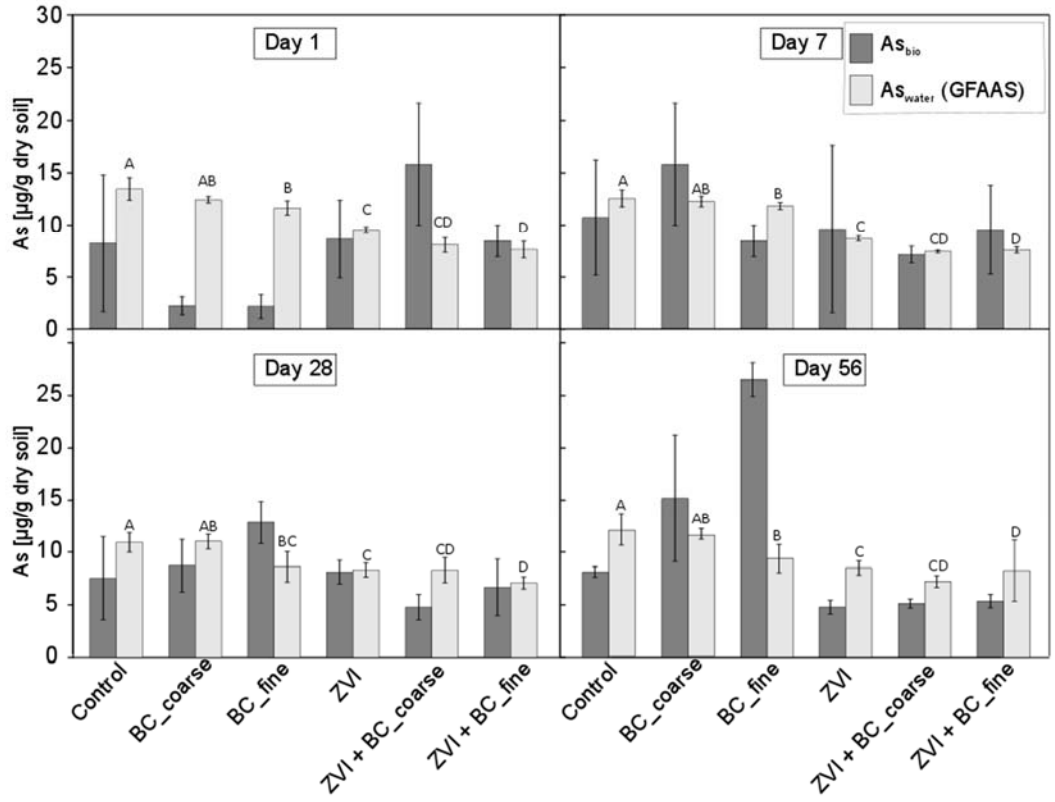

Figure S2: Comparison of As_{bio} and As_{ext} (measured with GF-AAS) at the different sampling times. (n=4, for control n=6; error bars represent standard deviation; different letters indicate statistically significant differences between the treatments ($p < 0.05$); statistical analysis was performed separately for As_{ext} and As_{bio}, meaning that different letters cannot be used for comparison between As_{ext} and As_{bio}). (BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron)

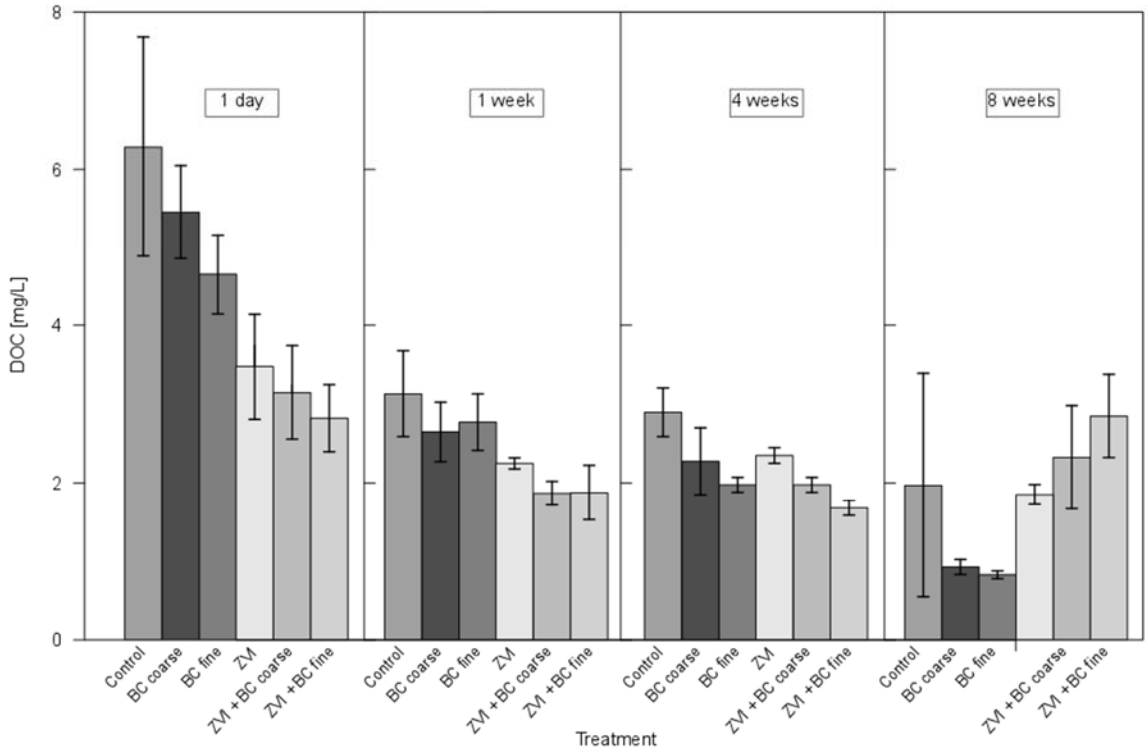

Figure S3: Dissolved organic carbon (DOC) in the water extracts (n=4, for control n=6, except ZVI (n=2) and ZVI + BC coarse (n = 3) at sampling time 1; error bars represent standard deviation) (BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron).

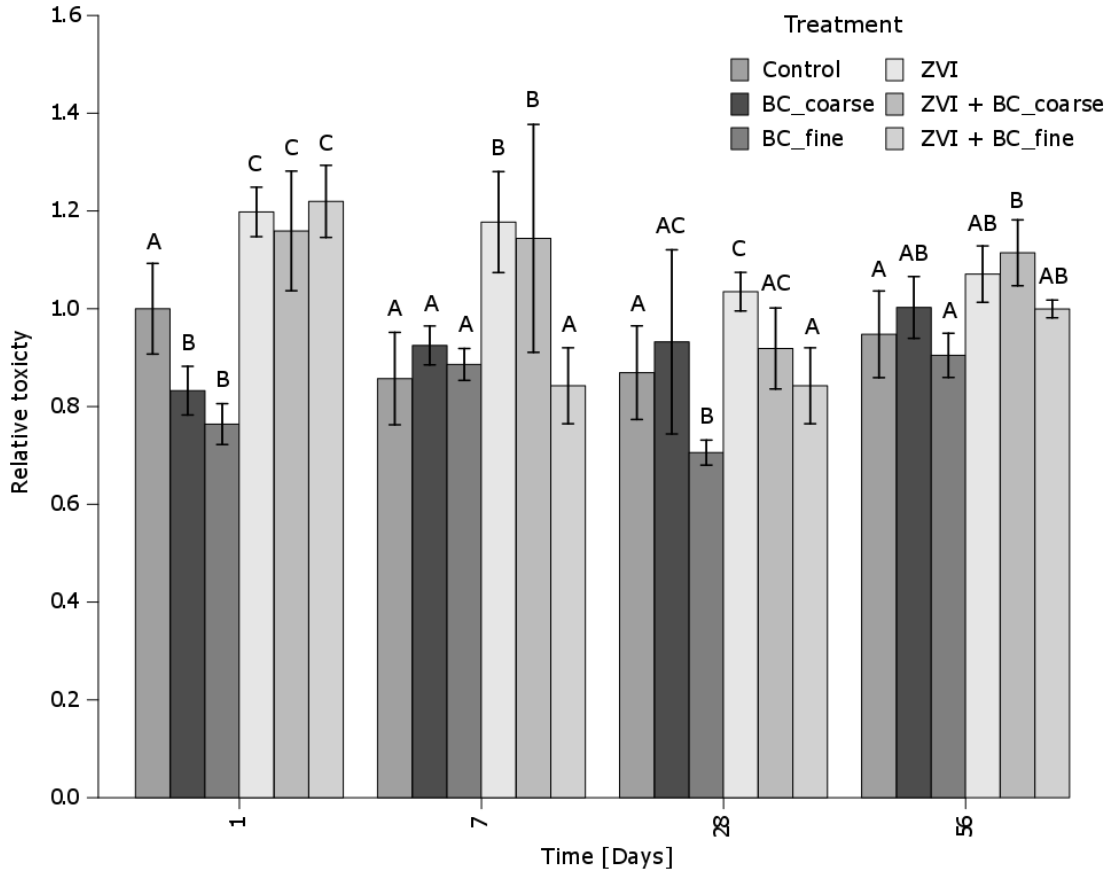

Figure S4: Relative toxicity, measured as bioluminescence from whole-cell bioreporter analysis with *E.coli* pUCD 607 HB101. Values are normalized by the mean of the control treatment at Day 1 with higher values indicating reduced toxicity (n = 4, for control n = 6; error bars represent standard deviation; different letters indicate statistically significant differences between treatments within one sampling time at $p < 0.05$). (BC = biochar; "coarse" and "fine" refer to different particle sizes of the biochar; ZVI = zero-valent iron)